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## Original Article

## Antioxidant activity and calcium binding of isomeric hydroxybenzoates

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## ABSTRACT

The association constant for calcium binding to hydroxybenzoates in aqueous 0.16 M NaCl at 25 °C was found electrochemically to have the value  $K_{\text{ass}} = 280 \text{ mol L}^{-1}$  with  $\Delta H^\circ = -51 \text{ kJ mol}^{-1}$ ,  $\Delta S^\circ = -122 \text{ J mol}^{-1} \text{ K}^{-1}$  for the 2-isomer (salicylate),  $K_{\text{ass}} = 7 \text{ mol L}^{-1}$  with  $\Delta H^\circ = -39 \text{ kJ mol}^{-1}$ ,  $\Delta S^\circ = -116 \text{ J mol}^{-1} \text{ K}^{-1}$  for the 3-isomer, and  $K_{\text{ass}} = 8 \text{ mol L}^{-1}$  with  $\Delta H^\circ = -51 \text{ kJ mol}^{-1}$ ,  $\Delta S^\circ = -155 \text{ J mol}^{-1} \text{ K}^{-1}$  for the 4-isomer. The 3- and 4-isomers were found more efficient as antioxidants than the 2-isomer in decreasing oxygen consumption rate in a peroxidating methyl linoleate emulsion and less sensitive to presence of calcium. All isomers were found prooxidative for iron-catalyzed initiation of oxidation due to enhanced radical formation as shown by electron spin resonance spectroscopy. Calcium salicylate was found to have low solubility with a solubility product  $K_{\text{sp}} = 4.49 \cdot 10^{-6}$  based on activity with  $\Delta H^\circ = 67 \text{ kJ mol}^{-1}$ ,  $\Delta S^\circ = 123 \text{ J mol}^{-1} \text{ K}^{-1}$  for dissolution in water, when corrected for the strong complex formation. Calcium in food and beverages may thus lower antioxidant activity of plant phenols through complexation or by precipitation.

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## 1. Introduction

Plant phenols are important nutrients and contribute to the health of fruit and vegetables and of beverage like coffee and tea [1–4]. Plant phenols are also important for the oxidative stability of many processed foods, as they are radical scavengers, absorbers of ultraviolet light and singlet oxygen quenchers [5–7]. Plant phenols interact together with carotenoids with redox-active metal ions like iron and copper inducing free radical processes under some conditions like during food digestion in effect becoming pro-oxidative [8–10].

Plant phenols, may, however, also interact with non-redox active metals like calcium, which could modify the capacity of the plant phenols as antioxidants or hamper their absorption from food during digestion. Such interaction has, however, been little studied despite the importance of such reactions for the stability of processed foods combining milk based component and plant material. We have selected one of the simplest plant phenols, hydroxybenzoic acid, and have studied the reactions of calcium with the three possible isomers of hydroxybenzoate, including salicylate, and report the effect of calcium on hydroxybenzoates as antioxidants.

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E-mail address: [ls@food.ku.dk](mailto:ls@food.ku.dk) (L.H. Skibsted).<http://dx.doi.org/10.1016/j.jfda.2017.07.001>1021-9498/Copyright © 2017, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Interaction between metal ions and antioxidants is receiving increasing attention [8]. Better methodologies for quantification of binding of metal ions to plant based antioxidants need, however, to be developed. The present study establishes such methods using a low molecular weight antioxidant in three isomeric forms combined with calcium as an important mineral nutrient.

## 2. Methods and materials

### 2.1. Materials

Calcium chloride dihydrate, sodium chloride, disodium hydrogenphosphate, sodium dihydrogenphosphate, iron(II) sulfate heptahydrate, sodium hydroxide, and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) were obtained from Merck (Darmstadt, Germany). Hydrogen peroxide (30%, v/v),  $\alpha$ -(4-pyridyl-N-oxide)-N-tert-butyl nitron (POBN) and ammonium purpurate 5,5'-nitroindibarbitoric acid (murexide indicator), methanol ( $\geq 99.9\%$ , v/v) were from Sigma–Aldrich (Steinheim, Germany). Sodium 2-hydroxybenzoate, 3-hydroxybenzoic acid, sodium 4-hydroxybenzoate, calcium salicylate, linoleic acid methyl ester, ascorbic acid, polyoxyethylenesorbitan monolaurate (Tween 20), and myoglobin from horse heart (min. 90%) were from Sigma (St. Louis, MO), while ethanol (96%, v/v) was from Kemetyl A/S (Køge, Denmark). All aqueous solutions were made from purified water using a Millipore Milli-Q purification system (Milli-Q Plus, Millipore Corporation, Bedford, MA).

### 2.2. Potentiometric determination of free calcium and association constants

A calcium ion-sensitive electrode ISE25CA together with a reference electrode REF 251 from Radiometer, Copenhagen, Denmark was used for measuring free calcium ion concentration in the presence of the hydroxybenzoate isomers at 0.0 °C, 10.0 °C, and 25.0 °C. The electrode was calibrated with calcium standard solutions of  $1.00 \times 10^{-4}$  mol L<sup>-1</sup>,  $1.00 \times 10^{-3}$  mol L<sup>-1</sup>, and  $1.00 \times 10^{-2}$  mol L<sup>-1</sup> before measurement. The standard solutions were prepared from a 1.00 mol L<sup>-1</sup> stock solution of dried CaCl<sub>2</sub> with the same ionic strength as the samples. NaCl was used for adjusting the ionic strength to 0.16 in standard solutions. The free calcium concentration of samples were determined from the linear

Nernst equation. A pH meter (713 pH Meter, Metrohm, Denmark) with a glass electrode (602 Combined Metrosensor glass electrode, Metrohm, Denmark) was used for measuring pH at ionic strength of 0.160 at 10.0 °C and 25.0 °C using activity pH standards for calibration.

### 2.3. Oxygen consumption rate

Two hundred and fifty microliter of 28.2 mM methyl linoleate (dissolved in methanol) was mixed with 63  $\mu$ L of 0.04 g mL<sup>-1</sup> Tween-20 dissolved in methanol. The methanol was then removed with a stream of nitrogen, and 2.50 mL of 5.0 mM air-saturated phosphate buffer (pH = 6.8) thermostatted at 25.0 °C was subsequently added. Ten microliter samples with a concentration of 0.00100 mol L<sup>-1</sup> of the hydroxybenzoate isomers with or without 0.0100 mol L<sup>-1</sup> calcium chloride were added. Twenty-five microliter of 0.20 mM myoglobin aqueous solution was added to the samples for initiating the oxidation. The mixed samples were injected into a thermostatted (25.0 °C) 70  $\mu$ L measuring cell (IKA-Labortechnik, Staufen, Germany) with no headspace. The Micro-respiration system–MRch System from Unisense (Århus, Denmark) was used to determine the oxygen consumption rate in the samples. A Clark electrode connected to a multichannel analyzer ReadOx-4H (Sable Systems, Henderson, NEV, USA) was used to measure the relative oxygen concentration of the samples in the measuring cells and data were recorded for 10 min at 10 s intervals. Before testing any samples, deoxygenated ascorbic acid mixed with sodium hydroxide corresponding to 0% oxygen and air-saturated phosphate buffer (100% oxygen corresponding to  $[O_2]_{\text{initial}} = 2.6 \times 10^{-4}$  mol L<sup>-1</sup>) thermostatted at 25.0 °C were used for electrode calibration [12].

The oxygen consumption rate  $V(O_2)$  in  $\mu\text{mol L}^{-1} \text{ s}^{-1}$  was calculated from:

$$V(O_2) = -\text{slope } [O_2]_{\text{initial}} \times 10^6/100 \quad (1)$$

The oxygen consumption rate  $V(O_2)$  was calculated based on the plot of oxygen percentage vs. time. The linear region of the curve (from 80% to 40%) was used to calculate the slope using linear regression analysis (% O<sub>2</sub> s<sup>-1</sup>) [13]. The antioxidative activity index value was used to compare the effect of the three hydroxybenzoate each with and without calcium addition on the rate of oxygen consumption:

$$\text{Antioxidative index} = \frac{\text{Oxygen consumption rate with antioxidant}}{\text{Oxygen consumption rate without antioxidant}} \quad (2)$$

relationship, based on the Nernst equation, between electrode potential (mV) and the corresponding calibration aqueous solution pCa value ( $-\log[Ca^{2+}]$ ) [11]. For determination of the solubility product of calcium salicylate in water, the calcium electrode was standardized using the same calcium standards but without sodium chloride addition and calcium activity calculated according to Davies equation. For both standardization methods, the calcium electrode responded according to

### 2.4. Fenton reaction with ESR detection of POBN spin adducts

A Miniscope Ms 200 Electron Spin Resonance (ESR) Spectrophotometer from Magnettech GmbH (Berlin, Germany) was calibrated with 2.0 mL POBN (3.2 M) in 1.0 M aqueous ethanol solution. Then, 2.0 mL POBN (3.2 M) in 1.0 M aqueous ethanol solution was added into the tube followed by addition of 10  $\mu$ L

FeSO<sub>4</sub> (0.022 M) and 25  $\mu$ L samples (0.045 mol L<sup>-1</sup> or 0.090 mol L<sup>-1</sup> of the hydroxybenzoate isomer) or Milli-Q water. The tube with Milli-Q water was used as reference. The reaction was initiated by addition of 40  $\mu$ L H<sub>2</sub>O<sub>2</sub> (0.024 M). Later, 50  $\mu$ L ESR micropipettes from Blaubrand IntraMark (Wertheim, Germany) were used for transferring the mixed sample solution to the resonator of the Miniscope MS 200 ESR spectrometer with following settings: Center field 336 G, sweep time 60 s, microwave power 10 mW as well as modulation amplitude 2000 mG. The data were recorded after 2 min. The two peak-to-peak amplitude seen in the ESR signal of the spin adducts were analyzed by Analysis 2.02 software program (ESR applications, Berlin, Germany).

The calculation of relative spin adduct fraction was based on:

$$I_{\text{ESR}} = \left(1 - \frac{\text{Peak height sample}}{\text{Peak height reference}}\right) \times 100\% \quad (3)$$

where the value of  $I_{\text{ESR}}$  quantify the reduction of the free radicals trapped by POBN in the presence of the hydroxybenzoate isomer [14].

### 2.5. EDTA titration

The EDTA titration solution with  $1.00 \times 10^{-2}$  mol L<sup>-1</sup> was standardized against  $1.00 \times 10^{-2}$  mol L<sup>-1</sup> CaCl<sub>2</sub>. One milliliter of sample was transferred to an Erlenmeyer flask and diluted to 15 mL with Milli-Q water. For maintaining the basic pH, 1.0 mL of 2.0 mol L<sup>-1</sup> NaOH was later added to each sample and 0.15 mL of 0.5% murexide solution was added as indicator. The titration end point was detected when the initial pink color changed to dark purple [15].

## 3. Results and discussion

The binding affinity between calcium and three hydroxybenzoate isomers was electrochemically determined at ionic strengths 0.16 at three different temperatures 0, 10, and 25 °C.

Association constant,  $K_{\text{ass}}$ , was used to quantify the binding affinity between the hydroxybenzoates, L<sup>-</sup>, and calcium in 1:1 complexes [16]:

$$K_{\text{ass}} = \frac{[\text{CaL}^+]}{[\text{Ca}^{2+}][\text{L}^-]} = \frac{C_{\text{Ca}^{2+}} - [\text{Ca}^{2+}]}{[\text{Ca}^{2+}](C_{\text{L}^-} - C_{\text{Ca}^{2+}} + [\text{Ca}^{2+}])} \quad (4)$$

In which,  $C_{\text{Ca}^{2+}}$  and  $C_{\text{L}^-}$  are total calcium and total hydroxybenzoate concentration, respectively, and  $[\text{Ca}^{2+}]$  is measured free calcium. According to the results presented in Table 1, the  $K_{\text{ass}}$  of three hydroxybenzoates decreased with increasing temperature indicating that



is an exothermic process for all three isomers of hydroxybenzoate in contrast to binding of calcium to hydroxycarboxylates in 1:1 complexes [16]. 2-Hydroxybenzoate, salicylate, binds calcium significantly stronger than the two other isomers for which the binding is weak and comparable in the investigated temperature interval. The constants were based on concentration and assuming formation of 1:1 complexes in agreement with previous studied carboxylates and are valid at physiological ionic strength [16].

The stronger binding of calcium to 2-hydroxybenzoate can be explained by a chelation effect, where a six-membered ring may be formed by interaction of the calcium ion with the lone pair of the phenolic oxygen, see Fig. 1. The binding of calcium to the hydroxy group does not, however, release a proton from the hydroxy group, as is evident from the only small decrease in pH seen, when calcium is added to a solution of 2-hydroxybenzoate. The decrease in pH following addition of calcium to 2-hydroxybenzoate is similar to the decrease in pH seen for 3-hydroxybenzoate and 4-hydroxybenzoate for which chelation is not possible and for which calcium solely binds to the carboxylate.

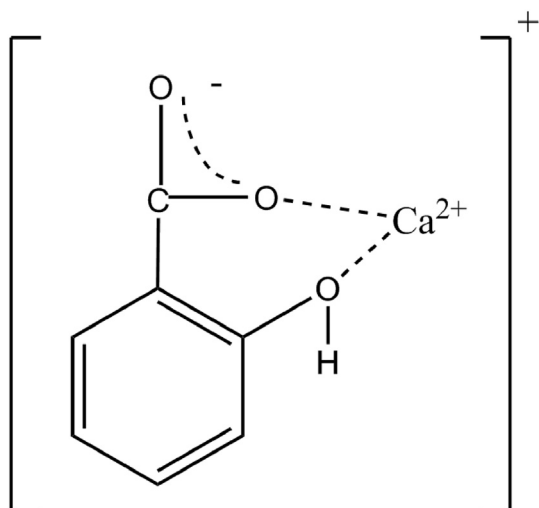
From the temperature dependence of the association constant the reaction enthalpy and reaction entropy were calculated according to

**Table 1 – Concentration based association constant and reaction enthalpy and entropy for complex formation for the 1:1 complexes between calcium and 2-hydroxybenzoate, 3-hydroxybenzoate and 4-hydroxybenzoate at 0.0 °C, 10.0 °C, and 25.0 °C in water with ionic strength 0.16 (NaCl).**

Ligand	Temperature (°C)	pH <sup>a</sup>	$K_{\text{ass}}$	$\ln K_{\text{ass}}$	$\Delta H^\circ$ (kJ mol <sup>-1</sup> )	$\Delta S^\circ$ (J mol <sup>-1</sup> K <sup>-1</sup> )	pKa <sup>b</sup>
2-Hydroxybenzoate	25	6.37					3.17
2-Hydroxybenzoate, Ca <sup>2+</sup>	0	—	1781 ± 39	7.48	-51 ± 5	-122 ± 19	
	10	6.34	973 ± 107	6.88			
	25	6.29	280 ± 3	5.63			
3-Hydroxybenzoate	25	7.18					4.08
3-Hydroxybenzoate, Ca <sup>2+</sup>	0	—	30 ± 10	3.40	-39 ± 0.5	-116 ± 2	
	10	6.95	16 ± 6	2.77			
	25	7.03	7 ± 1	1.95			
4-Hydroxybenzoate	25	6.93					4.58
4-Hydroxybenzoate, Ca <sup>2+</sup>	0	—	52 ± 13	3.95	-51 ± 8	-155 ± 30	
	10	6.93	31 ± 6	3.43			
	25	6.83	8.2 ± 0.2	2.08			

<sup>a</sup> pH in aqueous solution of each of the sodium salt of the three isomeric hydroxybenzoate without (25 °C) and with calcium chloride added (10, 25 °C).

<sup>b</sup> pKa value for corresponding hydroxybenzoic acid from reference [17].



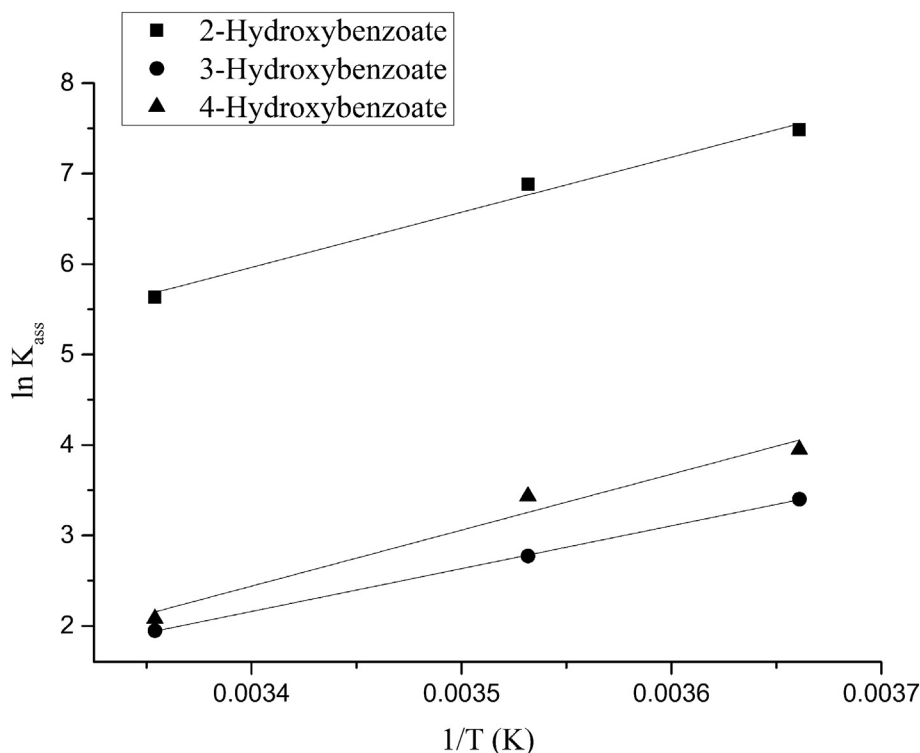
**Fig. 1** – Proposed binding of calcium to sodium 2-hydroxybenzoate.

$$-RT \ln K = \Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (6)$$

The van't Hoff plot for each of the three isomers of hydroxybenzoate, in agreement with the formula of eq. (6), is shown in Fig. 2, and the thermodynamic parameters are included in Table 1. All three complex formation processes are exothermic with  $\Delta H^0 < 0$ . For the 2- and 4-isomers of hydroxybenzoate,  $\Delta H^0 = -51 \text{ kJ mol}^{-1}$ , is more negative than

for the 3-isomer with  $\Delta H^0 = -39 \text{ kJ mol}^{-1}$ . The weak binding of calcium to the 4-isomers is an entropy effect, since  $\Delta S^0$  for binding of calcium to this isomer apparently increases ordering corresponding to the relative large negative  $\Delta S^0 = -155 \text{ J mol}^{-1} \text{ K}^{-1}$ . The weak binding of calcium to the 3-isomer is in contrast mainly an enthalpy effect. The pKa value for the isomeric hydroxybenzoic acids included in Table 1 shows that 2-hydroxybenzoic acid is a significantly stronger acid than the two other isomeric acids, an effect assigned to hydrogen bonding stabilizing the dissociated acid [17]. The stronger binding of calcium for the 2-hydroxybenzoate can be assigned to a similar effect of calcium binding between the carboxylate and the phenolic group.

The hydroxybenzoate were all found to be antioxidants in a peroxidating methyl linoleate oil-in-water emulsion. The rate of oxygen consumption is decreased for all isomers as is shown for 2-hydroxybenzoate in Fig. 3. The rate of oxygen consumption in presence of the hydroxybenzoates is affected by the presence of calcium corresponding to a decrease in antioxidant effect in the presence of calcium. The antioxidative index calculated from the oxygen consumption rate shows that 3-hydroxybenzoate and 4-hydroxybenzoate have a similar effect as antioxidant and both are more effective than 2-hydroxybenzoate, see Table 2. The antioxidative efficiency is seen to correlate with Bond Dissociation Energy (BDE) of the phenol oxygen–hydrogen bond, as BDE is larger for 2-hydroxybenzoate than for the two other isomers. A weak oxygen–hydrogen bond in a phenol (Ar–OH) promotes hydrogen atom transfer (HAT) in scavenging of lipid peroxyl radicals ( $\text{ROO}^\bullet$ ):



**Fig. 2** – van't Hoff plots for the concentration based association constant for calcium binding to 2-hydroxybenzoate, 3-hydroxybenzoate and 4-hydroxybenzoate in aqueous solution with ionic strength 0.16 (NaCl).

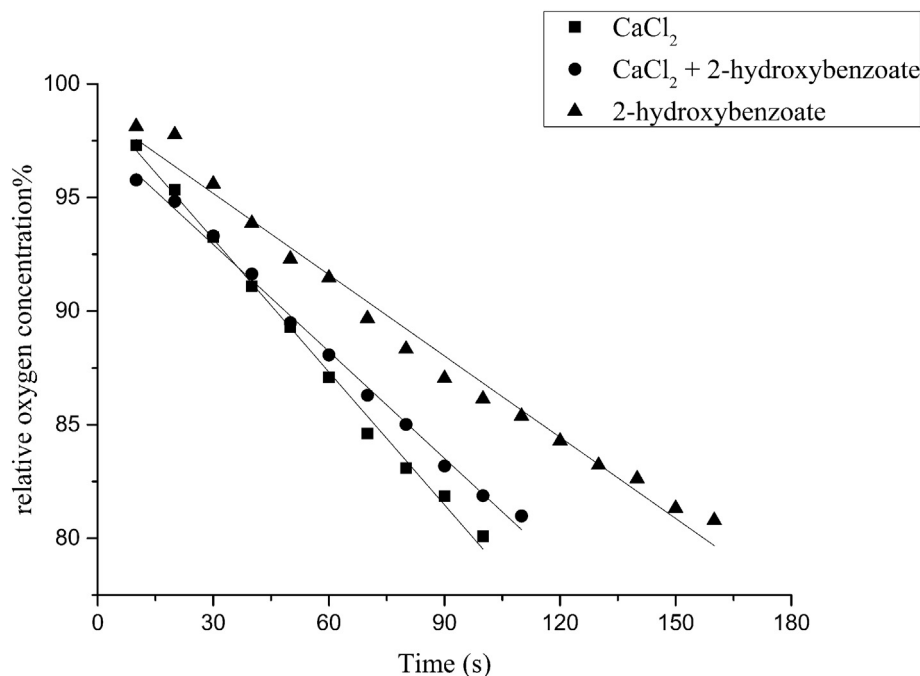


Fig. 3 – Rate of oxygen consumption in an aqueous methyl linoleate emulsion with calcium chloride ( $0.010 \text{ mol L}^{-1}$ ) added alone or with sodium 2-hydroxybenzoate ( $0.0010 \text{ mol L}^{-1}$ ) added alone or as their combinations determined electrochemically at  $25.0^\circ\text{C}$ . Full lines are linear regression lines.

Table 2 – Oxygen consumption rates for aqueous methyl linoleate emulsion for oxidation initiated by metmyoglobin in the presence of 2-hydroxybenzoate, 3-hydroxybenzoate, or 4-hydroxybenzoate with concentrations of  $0.0010 \text{ mol L}^{-1}$  in the absence or presence of  $0.010 \text{ mol L}^{-1}$   $\text{CaCl}_2$  determined electrochemically at  $25.0 \pm 0.1^\circ\text{C}$ . Average oxygen consumption rate for the relative oxygen concentration interval 80–40%.

Sample	Oxygen consumption rate ( $\mu\text{mol l}^{-1} \text{ s}^{-1}$ )	Antioxidative activity index <sup>a</sup>	BDE <sup>b</sup> $\text{kJ mol}^{-1}$
$\text{CaCl}_2$	$0.30 \pm 0.06$		
$\text{CaCl}_2 + 2\text{-hydroxybenzoate}$	$0.21 \pm 0.03$	0.71	401
2-Hydroxybenzoate	$0.18 \pm 0.06$	0.59	
$\text{CaCl}_2 + 3\text{-hydroxybenzoate}$	$0.16 \pm 0.01$	0.53	383
3-Hydroxybenzoate	$0.15 \pm 0.03$	0.50	
$\text{CaCl}_2 + 4\text{-hydroxybenzoate}$	$0.13 \pm 0.02$	0.43	392
4-Hydroxybenzoate	$0.14 \pm 0.01$	0.48	

<sup>a</sup> Antioxidative activity index relative to emulsion with  $\text{CaCl}_2$  added defined as ration between rates of oxygen consumption without and with hydroxybenzoate added.

<sup>b</sup> BDE is bond dissociation energy for oxygen–hydrogen bond in the phenolic group, as calculated by Density Functional Theory, from reference [20].

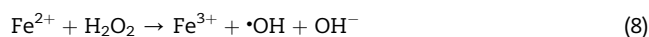


and accordingly, 2-hydroxybenzoate with the strongest oxygen–hydrogen bond is expected to be the least effective radical scavenger as confirmed by the experimental finding presented in Table 2.

Presence of calcium is expected to influence the antioxidative effect of the isomeric hydroxybenzoates. The effect was found to be largest for 2-hydroxybenzoate as seen from the experimental values for the antioxidative index presented in Table 2. Calcium increases the antioxidative index significantly for this isomer while smaller effects were detected for the 3- and 4-isomers in agreement with the weaker binding of calcium to these isomers. For 2-hydroxybenzoate, calcium is

bound closer to the phenolic group and with higher affinity in agreement with the more significant effect on the radical scavenging.

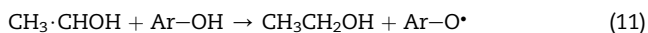
Oxygen consumption of peroxidating lipids relates to the propagation phase, during which antioxidants are active through radical scavenging. Some antioxidants are rather active by preventing formation of the free radicals initiating lipid and protein oxidation. The formation of free radicals is most directly detected by electron spin resonance spectroscopy [18]. One ESR method is based on spin trapping of hydroxylethyl radicals generated by the Fenton reaction





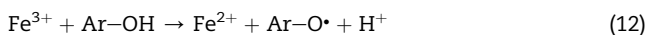


which many get trapped by 4-pyridyl-1-oxide-N-tert-butyl-nitron (POBN) or scavenged by the potential antioxidant



The POBN  $\text{CH}_3\cdot\text{CHOH}$  adduct is stable and may be quantified by ESR spectroscopy. A compound lowering the ESR signal of the POBN  $\text{CH}_3\cdot\text{CHOH}$  adduct is accordingly preventing initiation of oxidation, while a compound increasing the signal has a prooxidative effect. A prooxidative effect as seen for the isomeric hydroxybenzoates indicates that these compounds each are promoting radical formation in the Fenton reaction through reduction of iron(III) to iron(II).

The isomeric hydroxybenzoates are seen from the results presented in Table 3 to generate more trapped radical than generated alone by hydrogen peroxide indicating that the following reaction promotes radical generation

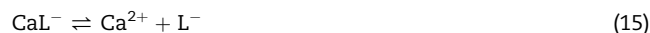


followed by the reactions of equations (8) and (9).

Calcium salicylate is only sparingly soluble in water, and the solubility in aqueous solutions of calcium salicylate was investigated from 0 to 25 °C. The solubility of calcium salicylate was determined from saturated aqueous solutions by determination of the total calcium by complexometric titration. Total calcium concentration corresponds to solubility of calcium salicylate. The solubility of calcium salicylate aqueous solution was increasing with increasing temperature as can be seen from Table 4. Solubility product of calcium salicylate was calculated using the activity of free calcium and hydroxybenzoate based on the value determined for  $K_{\text{ass}}$  of 2-hydroxybenzoate at 0 °C, 10 °C and 25 °C:

$$K_{\text{sp}}^{\text{activity}} = a_{\text{Ca}^{2+}} \cdot a_{\text{L}^-}^2 \quad (13)$$

The dissolution of calcium salicylate is a stepwise process, where  $\text{L}^-$  is the 2-hydroxybenzoate ions and  $\text{CaL}^+$  is the complex formed by calcium and 2-hydroxybenzoate:



The activity based association constant

$$K_{\text{ass}}^{\text{activity}} = \frac{a_{\text{CaL}^+}}{a_{\text{L}^-} \cdot a_{\text{Ca}^{2+}}} = \frac{\gamma_{\text{CaL}^+} \cdot [\text{CaL}^+]}{\gamma_{\text{L}^-} \cdot [\text{L}^-] \cdot a_{\text{Ca}^{2+}}} = \frac{[\text{CaL}^+]}{[\text{L}^-] \cdot a_{\text{Ca}^{2+}}} \\ = \frac{K_{\text{ass}}^{\text{concentration}}}{\gamma_{\text{Ca}^{2+}}} \quad (16)$$

was calculated from the concentration based constant using

$$a_{\text{Ca}^{2+}} = C_{\text{Ca}^{2+}} \cdot \gamma^{2+} \quad (17)$$

where the activity coefficient  $\gamma^{2+}$  was calculated from the Davies equation

$$\log \gamma^{2+} = -A_{\text{DH}} z^2 \left( \frac{\sqrt{I}}{1 + \sqrt{I}} - 0.30I \right) \quad (18)$$

where  $A_{\text{DH}}$  is the Debye–Hückel constant depending on temperature: 0.491, 0.498, and 0.510 at 0.0 °C, 10.0 °C, and 25.0 °C, respectively [19]. It is assumed that the activity coefficient for  $\text{L}^-$  and  $\text{CaL}^+$  is identical in agreement with Davies equation. The calculation was based on an iterative procedure calculating the ionic strength from the concentration of free ligand

$$[\text{L}^-] = C_{\text{Ca}^{2+}} + [\text{Ca}^{2+}] \quad (19)$$

and concentration of complex

**Table 3 – Relative effect of 2-hydroxybenzoate, 3-hydroxybenzoate, or 4-hydroxybenzoate on radical formation in oxidation of iron(II) by hydrogen peroxide (Fenton reaction) as measured by ESR spectroscopy using POBN as spin trap and expressed as index  $I_{\text{ESR}}$ . The negative values indicated prooxidative effects.**

Sample	$I_{\text{ESR}}$ (%) (hydroxybenzoate concentration 0.045 mol L <sup>-1</sup> )	$I_{\text{ESR}}$ (%) (hydroxybenzoate concentration 0.090 mol L <sup>-1</sup> )
2-Hydroxybenzoate	–51	–23
3-Hydroxybenzoate	–39	–44
4-Hydroxybenzoate	–60	–85

**Table 4 – Calcium concentration of a saturated aqueous solution of calcium salicylate as determined by EDTA titration together with activity based solubility product and reaction enthalpy and entropy for dissolution of calcium salicylate.**

Temperature (°C)	Total concentration of calcium (mol L <sup>-1</sup> )	$K_{\text{sp}}^{\text{activity}}$	$\ln K_{\text{sp}}^{\text{activity}}$	$\Delta H^\circ$ (kJ mol <sup>-1</sup> )	$\Delta S^\circ$ (J mol <sup>-1</sup> K <sup>-1</sup> )
0	0.054 ± 0.002	$(3.82 \pm 0.35) \cdot 10^{-7}$	–14.78	67 ± 4	123 ± 16
10	0.064 ± 0.0002	$(9.36 \pm 1.07) \cdot 10^{-7}$	–13.88		
25	0.0785 ± 0.0006 <sup>a</sup>	$(4.49 \pm 0.02) \cdot 10^{-6}$	–12.313		

<sup>a</sup> A value of 0.089 mol L<sup>-1</sup> was previously reported [21].

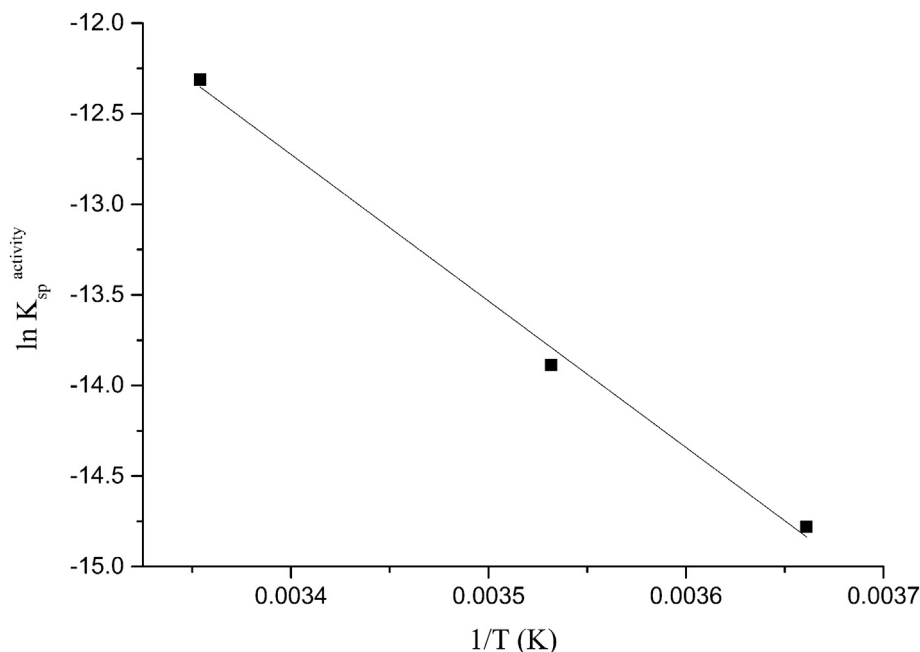


Fig. 4 – van't Hoff plot of the activity based solubility product of calcium salicylate in water.

$$[\text{CaL}^+] = C_{\text{Ca}^{2+}} - [\text{Ca}^{2+}] \quad (20)$$

$$I = \frac{1}{2} (4 \times [\text{Ca}^{2+}] + [\text{L}^-] + [\text{CaL}^+]) \quad (21)$$

The calculation procedure of ionic strength was repeated until the ionic strength value becomes stable as previously described [16]. With this stable ionic strength value, the values of  $a_{\text{Ca}^{2+}}$  was calculated from the calcium concentration, followed by  $[\text{CaL}^+]$  and  $[\text{L}^-]$  to yield the quadratic equation

$$K_{\text{ass}} \cdot [\text{Ca}^{2+}]^2 + (K_{\text{ass}} \cdot C_{\text{Ca}^{2+}} + 1) \cdot [\text{Ca}^{2+}] - C_{\text{Ca}^{2+}} = 0 \quad (22)$$

which was solved for  $[\text{Ca}^{2+}]$ .

From the obtained stable ionic strength value and the free calcium concentration, the activity of free calcium was calculated together with the activity of salicylate.  $K_{\text{sp}}$  for calcium salicylate for the three different temperatures was subsequently obtained and the results are summarized in Table 4 together with  $\Delta H^\circ$  and  $\Delta S^\circ$  for the dissolution process for calcium salicylate determined from the van't Hoff plot of Fig. 4. The dissolution process is endothermic and dissolution results in an increase in entropy in agreement with formation of ions from the solid compound.

Calcium is accordingly concluded to affect the antioxidative activity of simple plant phenols like the hydroxybenzoates. The influence of the structure could account for by differences between the three isomers in binding of calcium and by differences in bond dissociation energy of the three isomers as calculated by quantum mechanical methods. For the 2-hydroxy isomer, salicylate, also the low solubility of the calcium salt will affect the antioxidative activity. These studies will be extended to plant polyphenols like the catechins.

Interaction of calcium as an important mineral nutrient, often with low bioavailability, with dietary antioxidants is also important for mouth and teeth health [22]. Binding of calcium to proteins at their carboxylate side chain and to certain drugs may further affect their bioavailability [23,24]. The developed methods in the present study should be useful for future studies of such effects.

## Conflicts of interest

All authors declare no conflicts of interest.

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